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Applicants : Timothy V. Updyke et al.

For : SYSTEM FOR PH-NEUTRAL STABLE ELECTROPHORESIS GEL

EXPRESS MAIL CERTIFICATION

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I hereby certify that this transmittal letter and the other papers and fees identified in this transmittal letter as being transmitted herewith are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. § 1.10 on the date indicated above and are addressed to the Hon. Commissioner for Patents, Washington, D.C. 20231.


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TRANSMITTAL LETTER FOR RULE 53(b)
CONTINUING PATENT APPLICATION

Sir:

This is a request for filing a ☒ continuation, ☐ divisional, application of pending prior Application No. 09/228,875, filed January 11, 1999.

Transmitted herewith for filing are the ☒ specification; ☒ claims; ☒ abstract; ☒ declaration; ☒ power of attorney; for the above-identified patent application.

The enclosed declaration is:

- ☐ Newly executed (original or copy).
- ☒ Copy from a prior application (37 C.F.R. § 1.63(d)).
- ☐ A signed statement is attached deleting inventors named in the prior application (37 C.F.R. §§ 1.63(d)(2) and 1.33(b)).
- ☒ The entire disclosure of the prior application, from which a copy of the declaration is supplied, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

☒ The prior application, Application No. 09/228,875, filed January 11, 1999, is assigned of record to NOVEX, 11040 Roselle Street, San Diego, California 92121.

Also transmitted herewith are:

☐ _____ sheets of:

- ☐ Formal drawings.
- ☐ Informal drawings. Formal drawings will be filed during the pendency of this application.

☐ An assignment of the invention to _____

☐ A check in the amount of \$40.00 to cover the recording fee.

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☐ An associate power of attorney.

☐ A certified copy of the priority document, _____ application, No. _____, filed _____.

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Adam P. Noah

Adam P. Noah
Registration No. 45,601
Attorney for Applicants
Fish & Neave
Customer No. 1473
1251 Avenue of the Americas
New York, New York 10020-1104
Tel.: (650) 617-4000

defining pores which are saturated with an electrically conductive buffered solution of a salt. These pores through the gel are large enough to admit passage of the migrating macromolecules.

5 The gel is placed in a chamber in contact with buffer solutions which make electrical contact between the gel and the cathode or anode of an electrical power supply. A sample containing the macromolecules and a tracking dye is placed on top of
10 the gel. An electric potential is applied to the gel causing the sample macromolecules and tracking dye to migrate toward the bottom of the gel. The electrophoresis is halted just before the tracking dye reaches the end of the gel. The locations of the bands
15 of separated macromolecules are then determined. By comparing the distance moved by particular bands in comparison to the tracking dye and macromolecules of known mobility, the mobility of other macromolecules can be determined. The size of the macromolecule can
20 then be calculated.

 The rate of migration of macromolecules through the gel depends upon three principle factors: the porosity of the gel; the size and shape of the macromolecule; and the charge density of the
25 macromolecule. It is critical to an effective electrophoresis system that these three factors be precisely controlled and reproducible from gel to gel and from sample to sample. However, maintaining uniformity between gels is difficult because each of
30 these factors is sensitive to many variables in the chemistry of the gel system.

Polyacrylamide gels are commonly used for electrophoresis. Polyacrylamide gel electrophoresis or PAGE is popular because the gels are optically transparent, electrically neutral and can be made with a range of pore sizes. The porosity of a polyacrylamide gel is in part defined by the total percentage of acrylamide monomer plus crosslinker monomer ("%T") it contains. The greater the concentration, the less space there is between strands of the polyacrylamide matrix and hence the smaller the pores through the gel. An 8% polyacrylamide gel has larger pores than a 12% polyacrylamide gel. An 8% polyacrylamide gel consequently permits faster migration of macromolecules with a given shape, size and charge density. When smaller macromolecules are to be separated, it is generally preferable to use a gel with a smaller pore size such as a 20% gel. Conversely for separation of larger macromolecules, a gel with a larger pore size is often used, such as an 8% gel.

Pore size is also dependent upon the amount of crosslinker used to polymerize the gel. At any given total monomer concentration, the minimum pore size for a polyacrylamide gel is obtained when the ratio of total monomer to crosslinker is about 20:1, (the usual expression for this ratio would be "5%C").

Several factors may cause undesirable variation in the pore size of gels. Pore size can be increased by incomplete gel polymerization during manufacture. Hydrolysis of the polyacrylamide after polymerization can create fixed negative charges and break down the crosslinks in the gel, which will degrade the separation and increase the pore size. An

ideal gel system should have a reproducible pore size and no fixed charge (or at least a constant amount) and should be resistant to change in chemical characteristics or the pore size due to hydrolysis.

5 The size of the macromolecule varies between different macromolecules; the smaller and more compact the macromolecule the easier it will be for the macromolecule to move through the pores of a given gel. Given a constant charge density, the rate of migration
10 of a macromolecule is inversely proportional to the logarithm of its size.

For accurate and reproducible electrophoresis, a given type of macromolecule should preferably take on a single form in the gel. One
15 difficulty with maintaining uniformity of the shape of proteins during gel electrophoresis is that disulfide bonds can be formed by oxidation of pairs of cysteine amino acids. Different oxidized forms of the protein then have different shapes and, therefore, migrate
20 through the gel run with slightly different mobilities (usually faster than a completely reduced protein, since the maximum stokes radius and minimum mobility should occur with a completely unfolded form). A heterogeneous mixture of forms leads to apparent band
25 broadening. In order to prevent the formation of disulfide bonds, a reducing agent such as dithiothreitol (DTT) is usually added to the samples to be run. The shape of DNA and RNA macromolecules is dependent on temperature. In order to permit
30 electrophoresis on temperature-dependent DNA and RNA molecules in their desired form, separations are done at a controlled temperature.

The charge density of the migrating molecule is the third factor affecting its rate of migration through the gel -- the higher the charge density, the more force will be imposed by the electric field upon the macromolecule and the faster the migration rate subject to the limits of size and shape. In SDS PAGE electrophoresis, the charge density of the macromolecules is controlled by adding sodium dodecyl sulfate (SDS) to the system. SDS molecules associate with the macromolecules and impart a uniform charge density to them, substantially negating the effects of any innate molecular charge. Unlike proteins, the native charge density of DNA and RNA is generally constant, due to the uniform occurrence of phosphate groups. Thus, charge density is not a significant problem in electrophoresis of DNA and RNA.

SDS PAGE gels are usually poured and run at basic pH. The most common PAGE buffer system employed for the separation of proteins is that developed by Ornstein (1) and modified for use with SDS by Laemmli (2). Laemmli, U.K. (1970) Nature 227, 680-686. The Laemmli buffer system consists of 0.375 M tris (hydroxy methyl) amino-methane (Tris), titrated to pH 8.8. with HCl, in the separating gel. The stacking gel consists of 0.125 M Tris, titrated to pH 6.8. The anode and cathode running buffers contain 0.024 M Tris, 0.192 M glycine, 0.1% SDS. An alternative buffer system is disclosed by Schaeffer and von Jagow. Schaeffer, H. and von Jagow, G., Anal. Biochem. 1987, 166, 368-379. The stacking gel contains 0.75 M Tris, titrated to pH 8.45 with HCl. The separating gel contains 0.9 M Tris, titrated to pH 8.45 with HCl. The cathode buffer

contains 0.1 M Tris, 0.1M
N-tris(hydroxymethyl)methylglycine (tricine), 0.1% SDS.
The anode buffer contains 0.2 M Tris, titrated to pH
8.9 with HCl. For both of these systems Tris is the
5 "common ion" which is present in the gel and in the
anode and cathode buffers.

In the Laemmli system, the pH of the trailing
phase in the stacking gel is about 8.9. In the
separating gel, the trailing phase pH is about 9.7. At
10 this pH, primary amino groups of proteins react readily
with unpolymerized acrylamide, thiol groups are more
subject to oxidation to disulfides, or reaction with
unpolymerized polyacrylamide, than at neutral pH and
acrylamide itself is subject to hydrolysis.

15 The shape of the DNA and RNA macromolecules
is also dependent on a fourth important factor,
temperature. The temperature-dependent shape of DNA
and RNA is caused by the interaction of two
macromolecules containing complementary sequences and
20 the interaction of complementary sequences in a single
macromolecule. Some techniques require that the DNA
remain in its double-stranded form. Typically, such
separations are done in Tris borate ethylene diamine
tetra-acetic acid (TBE) buffer, consisting of 0.09 M
25 Tris, 0.09 M boric acid, and 0.002 M ethylene diamine
tetra-acetic acid (EDTA) on either polyacrylamide or
agarose gels. In general, these separations are done
at lower temperatures to maintain the double-stranded
structure. In the absence of denaturants, DNA's and
30 RNA's structure is fairly stable and not significantly
affected by temperature.

In other techniques, dissociation of the two DNA strands (known as "melting") is utilized to effect the separation. Such methods require careful temperature control in order to produce a consistent separation. One method, non-isotopic single-strand conformational polymorphism ("Cold SSCP"), utilizes a dissociative sample buffer with heat to melt the strands, a TBE buffer, and a polyacrylamide gel. In Cold SSCP, temperatures of 4 to 35°C are used to allow variable-conformation renaturation to occur between mutant strands, and temperature changes of only a few degrees can significantly alter the number of mutants seen. See Hongyo, et al., Nucleic Acids Research, 21, 3637 (1993). Another method, employed in DNA sequence analysis, typically utilizes TBE buffers containing 6 to 8 M urea and/or 2 to 12 M formamide, and elevated temperatures. It is important that the temperature remain high enough--typically 45 to 55°C--to maintain fully melted DNA or RNA. Gels are usually polyacrylamide and sometimes substituted acrylamide polymers. For example, certain alkyl-substituted polyacrylamide gels are described in Shoor et al., U.S. Patent 5,055,517.

These DNA and RNA separation methods are characterized by the use of continuous buffer systems, which use the same buffer species and generally, but not necessarily, in the same concentrations in the gel, the anode chamber, and the cathode chamber. These buffers usually are comprised of Tris and boric acid with EDTA added to inhibit hydrolytic enzyme activity. The TBE buffer system typically does not provide good stability when used in pre-cast gels, made and stored

for periods of weeks at 4°C. The polymer tends to break down, generating a fixed charge which leads to distortion particularly at the cathode end of the gel where resolution is especially important. Urea also
5 tends to break down under alkaline pH at 4°C. When large concentrations of urea are present, the ionic breakdown products can be present at a large enough concentration to disrupt the separation and cause loss of resolution.

10 Other buffer systems for DNA and RNA separations employ Tris/acetate, Tris/phosphate, and Tris/glycylglycine. While these buffer systems may be formulated near pH 7, the pK_a of Tris causes them to shift to an alkaline pH during electrophoresis
15 especially near the cathode. The applicants have found that the polyacrylamide and urea tend to break down during electrophoresis for DNA sequencing due to the high temperatures (50°C) employed for several hour runs when Tris is used as the buffering base. This
20 breakdown leads to higher current and lower resolution than might be obtained with a neutral pH buffer system, so that the DNA sequence read length is reduced and read errors are increased.

The need for uniformity and predictability is
25 magnified in precast electrophoresis gels which are manufactured by an outside vendor and then shipped to the laboratory where the electrophoresis will be performed. Precast gels must control the properties discussed above and they must be able to maintain this
30 control throughout shipping and storage. The shelf life of many precast gels is limited by the potential

for hydrolysis of acrylamide and/or buffer constitution during storage at the high pH of the gel buffer.

It is a disadvantage of a high pH gel that the polyacrylamide gel is subject to degradation by hydrolysis and has a limited shelf-life.

It is a further disadvantage of a high pH gel that proteins react readily with unpolymerized acrylamide which may interfere with subsequent analysis of the protein such as peptide sequencing.

It is a still further disadvantage of a high pH gel that thiol groups are subject to oxidation to disulfides causing a decreased resolution of separated macromolecules.

It is a further disadvantage of a high pH gel that buffer constituents such as urea break down readily.

Summary of the Invention

It is an object of this invention to produce a neutral gel system that reduces protein reaction with unpolymerized acrylamide thereby enhancing yield and resolution.

It is a further object of this invention to produce a neutral gel system that prevents formation of disulfides from free thiol groups thereby enhancing yield and resolution.

It is also an object of this invention to produce a neutral gel system that reduces degradation of the polyacrylamide gel by hydrolysis thereby increasing the stability of a gel during electrophoresis and the useful shelf-life of a precast gel, and better resolution.

It is also an object of this invention to produce a neutral gel system that reduces breakdown of buffer constituents, such as urea.

In accordance with this invention, applicants
5 describe a gel and buffer system wherein separation occurs at neutral pH and proteins remain completely reduced. Applicants also describe a gel and buffer system wherein storage of the gel and subsequent electrophoresis of macromolecules (such as DNA, RNA,
10 polypeptides and proteins) occurs at neutral pH. The above and other objects and advantages of the present invention will be apparent upon consideration of the following detailed description.

Detailed Description Of The Invention

15 Applicants describe a gel and buffer system wherein separation occurs at neutral pH and proteins remain completely reduced. Advantageously, at this neutral pH, primary amino groups of proteins react less readily with unpolymerized acrylamide because
20 protonation of protein amino groups greatly reduces their reactivity to acrylamide or other related blocking agents. Furthermore, at this neutral pH, thiol groups are less subject to oxidation than at higher pH and polyacrylamide itself is less subject to
25 hydrolysis.

The result is a gel system with improved stability of the gel matrix and stock solutions. Gels prepared according to this system can be stored under refrigeration for over a year without loss of
30 performance due to acrylamide hydrolysis. Also, stock buffers without reducing agents and stock gel solutions

without polymerization initiator can be stored for at least several weeks at room temperature with no loss of performance. An additional benefit is that a single gel recipe, using the same buffer for the stacking and
5 separating gels, can be used with two different running buffers to give two separation systems. Using this feature, an 8% gel, for example, can cover a protein separation range of 2 to 200 kDa.

In one embodiment of this invention a
10 polyacrylamide gel of between about 3% and about 25% (%T) acrylamide is polymerized using from about 1% to about 6% crosslinker (%C) using a gel buffer comprising a primary organic amine or substituted amine with a pK_a near neutrality, titrated with approximately half as
15 much HCl (on a molar basis), so that the pH of the buffer is approximately neutral. In a preferred embodiment the gel is polymerized using from about 2% to about 5% crosslinker (%C) using a gel buffer comprising bis-(2-hydroxyethyl) iminotris
20 (hydroxymethyl) methane (Bis-Tris) titrated with HCl. Different separation characteristics can be obtained by running the gel with either a 3-(N-morpholino) propanesulfonic acid (MOPS) or 2-(N-morpholino) ethanesulfonic acid (MES), buffer. 2mM to 10 mM
25 thioglycolic acid (TGA) or 2mM to 10mM sodium bisulfite is added to the running buffer to maintain a reducing environment in the gel during electrophoresis.

Applicants also describe another gel and buffer system for separation of macromolecules
30 (including DNA, RNA, polypeptides and proteins) wherein separation occurs at neutral pH. This gel and buffer system may be a discontinuous or continuous buffer

system, but is particularly useful in a continuous system. A continuous buffer system is one using the same buffer species and generally, but not necessarily, in the same concentrations in the gel, the anode chamber and the cathode chamber. This gel and buffer system permits higher resolution during electrophoresis when alkaline-labile compounds such as polyacrylamide and urea are present. This gel and buffer system also permits higher resolution when elevated temperatures are used. Advantageously, at this neutral pH, urea is less subject to hydrolysis. Furthermore, polyacrylamide itself is less subject to hydrolysis.

This gel and buffer system also possesses improved stability of the gel matrix and stock solutions. Gels prepared according to this system can be stored under refrigeration for over a year without loss of performance due to acrylamide hydrolysis. Also, stock buffers and stock gel solutions without polymerization initiator can be stored for at least several weeks at room temperature with no loss of performance.

In an embodiment of this gel and buffer system an electrophoresis gel is uniformly saturated with a gel buffer solution comprising a primary organic amine or substituted amine with a pK_a near neutrality, titrated with approximately an equimolar amount of acid or zwitterionic compound, so that the pH of the buffer is between about pH 6 and pH 8, preferably between about pH 6.5 to pH 7.5, and most preferably 6.5 to 7.0. The electrophoresis gel may be any agarose or polyacrylamide gel. Preferably, the electrophoresis gel comprises between 3% and 25% (%T) acrylamide

polymerized using from about 1% to about 6% cross linker (%C). More preferably, this polyacrylamide gel is polymerized using from about 2% to about 5% crosslinker (%C). Preferably, the amine comprises Bis-
5 Tris or N-(2-hydroxyethyl) morpholine, and most preferably, Bis-Tris. Suitable acids and zwitterionic compounds are hydrochloric acid, tricine, acetic acid, piperazine-N,N'-2-ethanesulfonic acid, 3-(N-morpholino)-propanesulfonic acid, 2-(N-morpholino)-
10 ethanesulfonic acid, N-(2-acetamido)-2-aminoethanesulfonic acid, 2-(N-morpholino)-2-hydroxypropanesulfonic acid, N-tris-(hydroxymethyl)-2-ethanesulfonic acid, N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid, N,N-bis-(hydroxyethyl)-2-
15 aminoethanesulfonic acid, and 3-(N-tris-(hydroxymethyl)methylamino)-2-hydroxypropanesulfonic acid. Tricine, 2-(N-morpholino)-ethanesulfonic acid, and piperazine-N,N'-2-ethanesulfonic acid are preferred for use in the buffer for a continuous gel and buffer system for
20 separation of DNA and RNA because the resulting system has separation characteristics similar to the commonly used TBE gel systems. Tricine is most preferred for that use. Preferably, the gel buffer comprises Bis-Tris titrated with tricine.

25 In a gel and buffer system, current increases and migration rates decline as the performance of the gels decline. The increase in current has been attributed to alkaline-catalyzed hydrolysis of urea present at 36% to 42% concentration. Any breakdown in
30 a neutral substance present at a large concentration, which produces a charged species will tend to disrupt the electrophoresis. This disruption arises from the

extra current produced, which in turn increases joule heating without aiding the separation. In addition, a discontinuity arises from the anionic and cationic hydrolysis products forming in the gel that are not present in the cathode and anode buffers. Hydrolysis of gel buffer species or additives takes place independently from the gel matrix composition. The decrease in migration rate may be attributed to higher fixed charge in the gel caused by alkaline-catalyzed hydrolysis of the gel's polyacrylamide. The fixed charge leads to significant counter-flow of water, which can retard a macromolecule's migration rate. It has been found that problems of gel instability producing lower resolution, increased current, decreased migration rates can be solved with gels buffered near neutrality and with buffer substances having a pK_a near neutrality. Such buffering systems improve the performance of fresh or pre-cast polyacrylamide gels, and fresh or pre-cast gels containing alkaline-labile materials, such as urea or formamide, even when the gels are made with base-stable polymers.

The inventors also have discovered the value of using different buffer substances in the cathode, gel, and anode buffers. A group of substitutions relating to cost and throughput have been discovered. The anionic substance used in the gel or the cathode buffer need not be present in the anode buffer since the anions do not migrate out of the anode buffer. In fact, the use of chloride or other strong acids in the anode buffer serves to increase the conductance of the buffer, thereby increasing the net voltage drop across

the gel and decreasing run times. Such acids are also typically much less expensive as compared to those employed in the cathode buffer. Similarly, the base used to adjust the pH of the cathode buffer need not be the same as that used in the gel. At a neutral pH, sodium hydroxide, Tris, and other organic bases with a basic pK_a have a higher conductance and often lower cost than Bis-Tris or other bases with a pK_a near neutrality. Using sodium or Tris salts in the cathode buffer will also decrease the gel run times. Often, the anode and cathode buffers are used at a higher concentration than in the gel, further increasing their conductance and decreasing gel run times. Thus, using different, more conductive anode and cathode buffer species than in the gel buffer increases throughput and decreases costs.

It was also found that Tris or Bis-Tris may be used in the anode buffer with no visible effect on the separation quality. Because of its higher pK_a , Tris gradually infiltrates the anode end of the gel increasing that region's conductance, causing the voltage drop to fall locally. Thus, the macromolecules near the anode slow down and the separation compresses, while the macromolecules near the cathode experience a higher voltage drop increasing their migration and relative separation. This effect of Tris actually improves the resolution of macromolecules at the cathode end of the gel where it is most needed. Tris is the preferred choice for routine use, because it is available at significantly lower cost than Bis-Tris and can improve read lengths.

The preferred embodiment of this invention uses Tris chloride in the anode buffer, the sodium or Tris salt of the acid or zwitterionic compound in the cathode buffer, and Bis-Tris as the gel buffer amine.

5 For protein and polypeptide separations, the most preferred cathode buffers are sodium or Tris salts of MOPS and MES, combined with a Bis-Tris chloride gel buffer. For DNA and RNA separations, the most preferred cathode buffers are sodium or Tris salts of
10 tricine, combined with a Bis-Tris tricine EDTA running buffer. When the buffer chambers are small, the most preferred molar concentrations of the cathode and anode buffers are five times that present in the gel buffer. These buffer systems provide the benefits of a neutral
15 pH gel during both storage and running, the least cost, and the fastest run times.

These and other embodiments can be understood by reference to the following illustrative and comparative examples.

20

Examples

Tris, Bis-Tris, MES, tricine, MOPS and Piperazine-N,N'-2-ethanesulfonic acid (PIPES) were purchased from Sigma (St. Louis, MO) or Research Organics (Cleveland, Ohio). Thioglycolic acid (TGA),
25 dithiothreitol (DTT) and beta-mercaptoethanol (BME) were from Sigma. All other chemicals were reagent, "ultra pure" or "electrophoresis grade" from standard sources.

In Example 1 through 6, gels were cast in 1
30 mm thickness mini-gel cassettes from NOVEX (San Diego CA) and run in an X-Cell minicell. The Bis-Tris

separating gel and stacking gels were prepared from a 30%T/2.5%C acrylamide/BIS stock solution and a 7X Bis-Tris stock solution (2.5M Bis-Tris, 1.5M HCl, pH 6.5). To prepare the separating gel, the stock solutions were
5 blended with ultra pure water to a final concentration 8%T, 0.357M Bis-Tris, to which was added 0.2 ul/ml N,N,N',N'-tetra-methyl-ethylene-diamine (TEMED). After degassing, 2.0 ul/ml of a 10% solution of ammonium persulfate (APS) was added, the gel was immediately
10 poured into the cassette then overlaid with water. Polymerization was allowed to proceed for at least 30 minutes at room temperature (RT), the water was removed and a 4% stacking gel applied. The stacking gel was prepared in the same fashion as the separating gel,
15 except that the final concentration obtained was 4%T, the TEMED concentration was increased to 0.4 ul/ml and the APS solution increased to 5.0 ul/ml. MOPS running buffer consisted of 50 mM MOPS, 50 mM Bis-Tris (or Tris), 0.1% SDS, 1 mM EDTA. MES running buffer
20 consisted of 50 mM MES, 50 mM Bis-Tris (or Tris), 0.1% SDS, 1 mM EDTA. Sample buffer (2X) consisted of 0.25 M Bis-Tris, 0.15 M HCl, 10% (w/v) Glycerol, 2% SDS, 1 mM EDTA, 0.03% Serva Blue G, and 200 mM DTT. Samples containing a set of protein standards were heated for
25 15 min at 70 degrees before application. Bovine serum albumin (BSA), chicken egg ovalbumin, alkylated insulin A and B chain, soybean trypsin inhibitor, and bovine erythrocyte carbonic anhydrase were included in the standard. Sample volume was 5 ul in all cases.

Example 1

The protein standards were separated on an 8% Bis-Tris/Cl gel with MOPS running buffer in the absence of a reducing agent. The resulting separation pattern
5 was very similar to that obtained on an 8% Tris/glycine gel (Laemmli), with proteins 20,000 and smaller remaining in the stack along with the tracking dye. The BSA band was somewhat diffuse and shifted toward the anode. The Ovalbumin band was also somewhat
10 diffuse.

Example 2

The protein standards were separated on an 8% Bis-Tris/Cl gel with MOPS running buffer in the presence of TGA in the cathode buffer. Again, the
15 separation pattern was very similar to that obtained on an 8% Tris/glycine (Laemmli) gel, with proteins 20,000 and smaller remaining in the stack along with the tracking dye. The presence of the reducing agent, 5 mM TGA, in the cathode buffer provided for better
20 resolution of the proteins BSA and Ovalbumin compared to the gel run without TGA.

Example 3

The protein standards were separated on an 8% Bis-Tris/Cl gel with MOPS running buffer in the
25 presence of sodium bisulfite in the cathode buffer. Again, the separation pattern was very similar to that obtained on an 8% Tris/glycine (Laemmli) gel, with proteins 20,000 and smaller remaining in the stack along with the tracking dye. The presence of the
30 reducing agent, 5 mM sodium bisulfite, in the cathode

buffer provided for better resolution of the proteins BSA and Ovalbumin compared to the gel run without sodium bisulfite.

Example 4

- 5 The protein standards were separated on an 8% Bis-Tris/Cl gel with MES running buffer in the absence of a reducing agent. The protein separation was very similar to that obtained from an 12% Tris/tricine (Schaegger) gel. All proteins were resolved from the
- 10 stack including insulin A and B chain (3500 and 2500 daltons, respectively). When the gel is run without TGA, soybean trypsin inhibitor had a more prominent doublet.

Example 5

- 15 The protein standards were separated on an 8% Bis-Tris/Cl gel with Bis-Tris/MES running buffer in the presence of TGA in the cathode buffer. Again, all proteins were resolved from the stack including insulin A and B chain (3500 and 2500 daltons, respectively).
- 20 The presence of the reducing agent, 5 mM TGA, in the cathode buffer provided for better resolution of the protein soybean trypsin inhibitor. Carbonic anhydrase ran as a tight, sharp band under all conditions tested.

Example 6

- 25 The protein standards were separated on an 8% Bis-Tris/Cl gel with Bis-Tris/MES running buffer in the presence of sodium bisulfite in the cathode buffer. Again, all proteins were resolved from the stack including insulin A and B chain (3500 and 2500 daltons,

respectively). The presence of the reducing agent, 5 mM sodium bisulfite, in the cathode buffer provided for better resolution of the protein soybean trypsin inhibitor. Carbonic anhydrase ran as a tight, sharp band under all conditions tested.

Although MES and MOPS were selected as desirable running buffers for protein separation because the resulting system has separation characteristics similar to the commonly used Laemmli and Schaeffer gel systems, it was found that a range of buffers are suitable for use in this system. Among the additional buffers giving good results were [N-(2-acetamido)]-2-aminoethanesulfonic acid (ACES), 2-[N-morpholino]-2-hydroxypropanesulfonic acid (MOPSO), N-Tris-(hydroxymethyl)-2-ethanesulfonic acid (TES), N,N-bis-(hydroxyethyl)-2-aminoethanesulfonic acid (BES), N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES), and 3-(N-Tris-(hydroxymethyl) methylamino)-2-hydroxypropanesulfonic acid (TAPSO).

All the proteins that exhibit some band broadening and/or mobility shifts when run in the absence of TGA or sodium bisulfite, have in common a composition that includes multiple cysteines (BSA, for instance, has 35 cysteines). On the other hand carbonic anhydrase, which always runs cleanly, has no cysteines. Moreover, if the reduced proteins are alkylated before running, they run as sharp homogeneous bands even in the absence of a reducing agent.

Cysteine-containing proteins appear to give generally sharper bands in the Laemmli system than the neutral system, when both are run with 100 mM mercaptoethanol or DTT in the sample buffer but without

TGA in the running buffer. Since thiol oxidation is more favored as the pH increases, it would be expected that the higher pH of the Laemmli system would cause oxidation of disulfide to be at least as pronounced as
5 it is in the neutral pH system. However, DTT and similar "neutral" thiol reducing agents are weak acids (with pK_a 's around pH 8-9). Thus, at basic pH, these reducing agents migrate into the gel and, if present at sufficient concentration, provide some protection
10 against oxidation of sulfhydryls. At a neutral separating pH, DTT from the sample buffer is in an uncharged form and will remain behind in the sample well. Thus, no reducing agent migrates into the gel.

To maintain proteins in a reduced form during
15 electrophoresis at neutral pH, it was found advantageous to use a reducing agent that would migrate into the gel at neutral pH. Sodium bisulfite (2-10 mM) was found to maintain a reducing environment in the gel during electrophoresis. Fully reduced TGA (or similar
20 negatively charged thiols) give similar results at comparable concentrations. However, partially oxidized TGA will promote partial oxidation of protein thiols. Because reduction (oxidation) of protein thiols will take place via disulfide interchange, the ratio of
25 reduced to oxidized thiols in the protein will substantially reflect the ratio of reduced to oxidized thiols in the TGA. Conversely, sulfite oxidizes to sulfate, which does not participate in redox reactions under conditions found in the gel. Therefore,
30 regardless of the sulfite/sulfate ratio in a partially oxidized preparation of sulfite, as long as sufficient

sulfite remains, proteins will be protected against thiol oxidation.

It was also found that Tris could be substituted for Bis-Tris in the running buffer with no visible effect on the separation quality. Bis-Tris may be preferred where the protein will be intentionally modified post-separation. Bis-Tris is a tertiary amine and will not interfere with the protein modifying agents which react through primary amines. Tris, however, is the preferred choice for routine use, because it is available at significantly lower cost than Bis-Tris.

Example 7

A 14.7%T/5%C TBE urea gel was made in the following manner. To prepare the separating gel solution, a 30% acrylamide/ 1.6% bis-acrylamide stock solution (47.5 ml), and a 5X gel buffer stock solution containing 0.45 M Tris, 0.45 M boric acid, and 0.01 M EDTA, pH 8.18 (20 ml) were mixed with urea (36 g), TEMED (20 ul), and enough water was added to make 100 ml. The final solution pH was 8.87. It was degassed, a 10% ammonium persulfate solution ("10% APS") (12.8 ul) was added to 6.4 ml, and poured into a 1 mm thick mini-gel cassette from NOVEX (San Diego, CA). A comb-forming gel solution was made similarly, with the following differences: acrylamide/bis solution (12.7 ml), TEMED (50 ul), and no urea; to 1.0 ml of this solution was added 10% APS (0.1 Ml) and it was immediately poured on top of the separating gel solution. A 1 mm 10-well comb (NOVEX) was added, and the gels were allowed to polymerize for at least 30

minutes at room temperature. They were then run after storage in sealed pouches with 1X gel buffer containing 7M at different temperatures.

The gels were run fresh or after storage at
5 either 4°C or 35°C. Samples employed were a 10b Oligo DNA standard (BRL, Bethesda, MD) or an 18-mer custom-synthesized DNA fragment (Synthetic Genetics, San Diego, CA). Gels were run in an X-Cell mini-cell (NOVEX) at 180 volts for 80 minutes, using 1X gel
10 buffer in both the anode and cathode chambers. Finally, the bands were visualized by treating with Stains-All solution (Sigma) for 15 minutes then destaining in 20% methanol for 10 minutes.

Compared to fresh gels, the gels stored at
15 4°C showed a gradual loss of band sharpness and an increase in current during the electrophoresis. The loss of sharpness leads to less resolution between bands. After 2 weeks at 4°C, the band width had doubled as compared to fresh gel bands. When stored at
20 35°C for 1 week, the gels ran with higher current but the dye front only migrated 80% as far in 80 minutes. The gel itself retained the stain, and the bands were fuzzy and indistinct. After three weeks, no bands could be seen and the gels were very fragile.

25

Example 8

Gels were prepared, stored, and run as described in Example 7, except that the 5X gel buffer was composed of 0.45 M Bis-Tris, 0.45 M tricine, and 0.01 M EDTA pH 7.27, the final gel solution pH was
30 7.70, and the running buffer was 0.05 M Tris, 0.05 M tricine, 0.001 M EDTA. These gels showed no

significant change in band sharpness, running current, or migration distances when stored for up to 3 weeks at 35°C or for several months at 4°C.

Example 9

5 Gels were prepared, stored, and run as described in Example 7, except that the 5X gel buffer was composed of 0.125 M N-(2-hydroxyethyl) morpholine (HEM), 0.083 M acetic acid, and 0.002 M EDTA pH 7.0, and the final gel solution pH was 7.21. These gels
10 showed no significant change in band sharpness, running current, or migration distances when stored for up to three weeks at 35°C or for several months at 4°C. However, the gels turned yellow on storage at 35°C.

Example 10

15 Mini-DNA sequencing gels were prepared from the same separating gel solution as described in Example 7, except that only 23.3 ml of acrylamide/bis solution was employed, the urea was increased to 42 g, and the TEMED was increased to 50 ul. It was used
20 without degassing by adding 10% APS (50 ul) to 10 ml of the solution, and pouring between 11 cm wide by 22 cm long thick glass plates with 0.25 mm spacers. The gels were allowed to polymerize for 60 minutes at room temperature, then run the same day.

25 Samples employed were an M-13 DNA sequencing reaction prepared with S³⁵-label using a USB Sequenase kit, version 2, (United States Biochemicals, Cleveland, OH). They were run in a custom-made DNA sequencing chamber at 15 watts (about 50°C). Finally, the bands
30 were visualized by autoradiography. The gels had a

read length of 120 bases with a 5% error rate (95% accuracy).

Example 11

Gels were prepared and run as in Example 10,
5 except that the 5x gel buffer was composed of 0.5 M Bis-Tris, 0.84 M tricine, and 0.01 M EDTA pH 7.2 and the final gel solution pH was 7.50. The gels had a read length of 137 bases with a 1.5% error rate (98.5% accuracy).

Example 12

10 The separating gel solution was prepared as in Example 7, except that a SoaneGel SQ solution (a solution of substituted acrylamide and substituted bis-acrylamide cross-linkers, available from Soane
15 Biosciences Inc., Hayward, CA) was used for the polymer at 6%T, and the TEMED was increased to 88 ul. After initiation of the gel solution (40 ml) with 10% APS (200 ul), the gels were poured in plates with 0.35 mm spacers for an ABI Model 377 DNA Sequencer (Applied
20 Biosystems Division of Perkin Elmer Corp., Foster City, CA), and allowed to polymerize at room temperature for 2 hours. The gels were loaded with a pGEM sequencing reaction and run with 1X TBE buffer at 30 V/cm, generating 55°C. They had a read length of 815 bases;
25 at 550 bases the error rate was 1.5% (98.5% accuracy).

Example 13

Gels were prepared and run as in Example 12, except that the 5X gel buffer was composed of 0.5 M Bis-Tris, 0.84 M tricine, and 0.01 M EDTA pH 7.2, and

the final gel solution pH was 7.5. The gels were run with 1X gel buffer at 30 V/cm, generating 55°C, and had a read length of 866 bases; at 550 bases the error rate was 1.1% (98.9% accuracy).

- 5 Although the invention has been explained in relation to its preferred embodiments, it is to be understood that various modifications thereof will become apparent to those skilled in the art. The foregoing disclosure is not intended or to be construed
- 10 to limit the present invention, or to otherwise exclude any such other embodiments, adaptations, variations and equivalent arrangements, the present invention being limited only by the claims appended hereto and the equivalents thereof.

We claim:

1. A discontinuous buffer gel electrophoresis system comprising:

an electrophoresis gel suitable for casting, the electrophoresis gel uniformly saturated with a gel buffer comprising an organic amine with a pK_a near neutrality and an acid, the gel buffer having a pH between 5.5 and 7.5; and

a cathode buffer comprising an antioxidant that is anionic at neutral pH in a concentration sufficient to maintain proteins in a reduced state.

2. The system of claim 1, wherein the organic amine is Bis(2-hydroxyethyl) iminotris(hydroxymethyl) methane.

3. The system of claim 1, wherein the electrophoresis gel is a polyacrylamide gel.

4. The system of claim 1, wherein the acid is selected from the group consisting of hydrochloric acid and acetic acid.

5. The system of claim 1, wherein the antioxidant comprises sulfite.

6. The system of claim 1, wherein the antioxidant comprises bisulfite.

7. The system of claim 1, wherein the antioxidant comprises a thiol.

8. The system of claim 1, wherein the antioxidant comprises thioglycolic acid.

9. The system of claim 1, wherein the gel buffer further comprises a dodecyl-sulfate salt.

10. The system of claim 9, wherein the dodecyl-sulfate salt is sodium dodecyl sulfate.

11. The system of claim 1, wherein the cathode buffer further comprises 3-(N-morpholino) propanesulfonic acid.

12. The system of claim 1, wherein the cathode buffer further comprises 2-(N-morpholino) ethanesulfonic acid.

13. A discontinuous buffer gel electrophoresis system comprising:

an electrophoresis gel suitable for casting, the electrophoresis gel uniformly saturated with a gel buffer comprising an organic amine with a pK_a near neutrality and an acid, the gel buffer having a pH between 5.5 and 7.5; and

a cathode buffer comprising an antioxidant that migrates into the gel by electrophoresis and that has a concentration sufficient to maintain proteins in a reduced state.

14. A discontinuous buffer gel electrophoresis system comprising:

an electrophoresis gel suitable for casting, the electrophoresis gel uniformly saturated with a gel buffer comprising an organic amine with a pK_a near neutrality and an acid, the gel buffer having a pH between 5.5 and 7.5; and

a cathode buffer comprising 3-(N-morpholino) propanesulfonic acid.

15. A discontinuous buffer gel electrophoresis system comprising:

an electrophoresis gel suitable for casting, the electrophoresis gel uniformly saturated with a gel buffer comprising an organic amine with a pK_a near neutrality and an acid, the gel buffer having a pH between 5.5 and 7.5; and

a cathode buffer comprising 2-(N-morpholino) ethanesulfonic acid.

16. A method for performing electrophoresis using a discontinuous buffer gel, the method comprising:

providing an electrophoresis gel suitable for casting;

uniformly saturating the electrophoresis gel uniformly with a gel buffer comprising an organic amine with a pK_a near neutrality and an acid, the gel buffer having a pH between 5.5 and 7.5; and

providing a cathode buffer comprising an antioxidant that is anionic at neutral pH in a

concentration sufficient to maintain proteins in a reduced state.

17. The method of claim 16, wherein the organic amine is Bis(2-hydroxyethyl) iminotris(hydroxymethyl) methane.

18. The method of claim 16, wherein the electrophoresis gel is a polyacrylamide gel.

19. The method of claim 16, wherein the acid is selected from the group consisting of hydrochloric acid and acetic acid.

20. The method of claim 16, wherein the gel buffer further comprises a dodecyl-sulfate salt.

21. The method of claim 20, wherein the dodecyl-sulfate salt is sodium dodecyl sulfate.

22. A method for performing electrophoresis using a discontinuous buffer gel, the method comprising:

providing an electrophoresis gel suitable for casting;

uniformly saturating the electrophoresis gel with a gel buffer comprising an organic amine with a pK_a near neutrality and an acid, the gel buffer having a pH between 5.5 and 7.5; and

providing a cathode buffer comprising an antioxidant that migrates into the gel by

electrophoresis and that has a concentration sufficient to maintain proteins in a reduced state.

ABSTRACT

A gel and buffer system for gel electrophoresis wherein separation occurs at neutral pH.

**DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

SYSTEM FOR PH-NEUTRAL STABLE ELECTROPHORESIS GEL

the specification of which

(check ☒ is attached hereto
one)

[] was filed on _____ as
Application Serial No. _____
and was amended on _____.
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I do not know and do not believe that the invention was ever patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application.

I do not know and do not believe that the invention was in public use or on sale in the United States of America more than one year prior to this application.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known by me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

| | | | <u>Priority Claimed</u> | |
|-----------------|------------------|-------------------------------|--------------------------|--------------------------|
| | | | <input type="checkbox"/> | <input type="checkbox"/> |
| <u>(Number)</u> | <u>(Country)</u> | <u>(Day/Month/Year Filed)</u> | Yes | No |
| <u>(Number)</u> | <u>(Country)</u> | <u>(Day/Month/Year Filed)</u> | <input type="checkbox"/> | <input type="checkbox"/> |
| <u>(Number)</u> | <u>(Country)</u> | <u>(Day/Month/Year Filed)</u> | Yes | No |
| <u>(Number)</u> | <u>(Country)</u> | <u>(Day/Month/Year Filed)</u> | <input type="checkbox"/> | <input type="checkbox"/> |
| <u>(Number)</u> | <u>(Country)</u> | <u>(Day/Month/Year Filed)</u> | Yes | No |

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known by me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

| | | |
|--------------------------|-------------------------|---|
| <u>08/221,939</u> | <u>March 31, 1994</u> | <u>Patented</u> |
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| (Application Serial No.) | (Filing Date) | (Status) (patented, pending, abandoned) |

As a named inventor, I hereby appoint the following attorneys or agents to prosecute this application and transact all business in the United States Patent and Trademark Office connected therewith:

Edward J. DeFranco, Esq. - Reg. No. 39,653

Nicola A. Pisano, Esq. - Reg. No. 34,408

James Trosino, Esq. - Reg. No. 39,862

Send correspondence to: James Trosino
FISH & NEAVE
1251 Avenue of the Americas
New York, New York 10020

Direct telephone calls to: James Trosino
(650) 617-4000

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first inventor Timothy V. Updyke


First Inventor's signature *Timothy V. Updyke* 06/14/99
Date

Residence 37349 Avenida Chapala, Temecula, California 92592

Citizenship United States of America

Post Office Address 37349 Avenida Chapala

Temecula, California 92592

Full name of second joint inventor Sheldon C. Engelhorn
Second Inventor's signature  Jan 6, 1999
Date
Residence 2474 Manchester Avenue,
Cardiff-by-the-Sea, California 92007
Citizenship United States of America
Post Office Address 2474 Manchester Avenue
Cardiff-by-the-Sea, California 92007